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A direct, quantitative polymerase chain reaction (PCR) assay was employed to measure the levels of gag DNA and geonomic RNA in cryopreserved patient peripheral blood mononuclear cells (PBMC). This technique was used to determine the viral load over the initial 702 day period (range: 492-812 days) in 26 of the volunteer receipients of a recombinant gp 160 vaccine in a Phase I safety trial. We observed no statistically significant alterations in viral load over the study period. In addition, the assessment at multiple time points post immunization, of the first three patients to enter the Phase I trial revealed no statistically significant relationship between immunization and viral load.

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Assessment of *gag* DNA and Genomic RNA in Peripheral Blood Mononuclear Cells in HIV-Infected Patients Receiving Intervention with a Recombinant gp160 Subunit Vaccine in a Phase I Study

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HUMAN IMMUNODEFICIENCY VIRUS disease progression is characterized by a period of high viremia during acute infection, followed by host immune response concomitant with a decline in viremia,[1–3] and by a final phase of high viremia late in disease.[1,3] During the protracted clinically asymptomatic period, while virus may be difficult to recover in culture,[4,5] viral sequences can be detected by the polymerase chain reaction (PCR), suggesting that the disease is characterized by a persistent level of viral expression throughout this period.[3,6–12]

Refinement of the techniques of PCR and reverse transcriptase-linked polymerase chain reaction (RT-PCR) assays by our laboratory enabled us to investigate the relationship between increases in viral DNA and RNA load in 31 patients assessed in a cross-sectional study and representing all stages of HIV infection.[8] To complement the characterization of the immune responses of vaccine recipients to the recombinant gp160 subunit vaccine described in Redfield *et al.*,[13] we initiated the present study, employing PCR to assess proviral (*gag*) DNA and RT-PCR to assess genomic RNA production, in patients receiving the vaccine in a phase I trial.

Vaccine recipients were participants in a phase I safety and immunogenicity trial of a recombinant gp160 vaccine. Patients were characterized as seropositive, Walter Reed stage 1 or 2,[14] with a mean of no fewer than 400 $CD4^+$ cells/mm^3 for a greater than 3-month period prior to entry in the trial and were described as having responded (patients A, C, E, F, G, H, I, K, L, M, O, P, R, U, V, X, and Y) or not responded (patients B, D, J, N, Q, S, T, W, and Z) to the initial vaccine regimen according to the immunological criteria defined by Redfield *et al.*[13] The demographic information in Table 1 summarizes patient profiles, sampling points, and the timing of immunizations. In many patients, no immunizations were received between the completion of the initial immunization series on either day 120 or 180 until day 400–600 of follow-up, leaving washout periods of 220–480 days. All the initial priming shots were variable doses; repriming consisted of 640 μg (six shots) and boosting of 160 μg per dose. The vaccine is a subunit gp160 recombinant protein expressed in baculovirus and biochemically purified and adsorbed to aluminum phosphate and is manufactured by MicroGeneSys (Meriden, CT) under the trade name VaxSyn.

For PCR, approximately 5×10^6 patient peripheral blood mononuclear cells (PBMCs) were thawed on ice, pelleted at 1500 rpm at 4°C for 5 min washed once with cold phosphate-buffered saline (PBS), and resuspended in a final volume of 5.0 ml of cold PBS. DNA was prepared from 1 ml of washed cells, employing detergent lysis followed by digestion with proteinase K (Bethesda Research Laboratories, Gaithersburg, MD). RNA was prepared from 4 ml of washed cells, using extraction in 4 M guanidine isothiocyanate, 0.3 M sodium acetate (pH 6.0), and 0.3 M 2-mercaptoethanol, followed by cesium chloride gradient centrifugation. RNA was resuspended in 100 μl of diethyl pyrocarbonate (DEPC)-treated water prior to treatment with 135 U of RNase-free DNase I (Boehringer Mannheim, Indianapolis, IN).

The sequences of the primer pairs used in PCR and RT-PCR, for *gag* DNA and genomic RNA, were as follows:

GAG-3: CAATGAGGAAGCTGCAGAATGGGATAG
GAG-6: CATCCATCCTATTTGTTCCTGAAGG
Probe, GPR-5: ATGAGAGAACCAAGGGGAAGTGACATAGCA

For *gag* DNA PCR, 100,000 cell equivalents were used in a 100-μl reaction employing 25 cycles and an annealing temperature of 55°C. For genomic RNA PCR, RNA was converted to cDNA in a 20-μl reaction volume incubated at 45°C for 1 hr using 8 ng of template RNA in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, cloned RNase H-Moloney murine leukemia virus reverse transcriptase (10 U/μl) (SuperScript; Bethesda Research Laboratories), RNasin (1 U/ml; Promega, Madison, WI), 20 mM dithiothreitol (DTT), 0.5 mM dNTPs, and 0.5 μM 3'

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TABLE 1. PATIENT PROFILES IN GP160 PHASE I TRIAL[a,b]

Patient	Sex	Ethnicity	Age at entry (years)	No. of injections primary	Initial responder[a]	Reprime[b]	Boosters[c]	Day to C/H[d]	AZT use[e]	Length of follow-up[f]
A	M	B	24	6	R		4	45		807
B	M	W	24	3	N	611	0	640		807
C	M	B	35	6	R		3	150		812
D	M	W	34	6	N	617	0	729		645
E	M	B	25	6	R		3	120		749
F	M	B	41	6	R		4	150	685–699	803
G	M	W	25	6	R		2	75		774
H	M	B	24	3	R		5	60		730
I	M	B	23	6	R		3	90		783
J	M	B	25	3	N	615	0	672		811
K	M	B	32	3	R		5	180		643
L	M	B	27	6	R	512	1	180		755
M	M	B	28	3	R		6	180		734
N	M	H	29	3	N	372	1	483		623
O	F	B	38	6	R		2	150	515–1100	586
P	M	W	27	3	R		5	150		731
Q	M	W	43	3	N	559	0	672	600–	755
R	F	W	48	6	R		4	180		745
S	M	C	36	6	N	316	2	429	610–1160	758
T	M	W	49	3	N	301	2	441		732
U	M	W	26	6	R	279	2	421		735
V	F	W	19	6	R		4	90		732
W	M	B	27	3	N	288	1	400		568
X	F	H	22	6	R		5	90		800
Y	M	W	27	6	R	249	2	90		675
Z	M	B	29	3	N	268	0	120		492
5[g]	M	W	30	3	N					
11[g]	M	H	27	6	R		1	120		
12[g]	M	W	27	3	N	681	0			
22[g]	M	W	21	3	R					
HIV positive serum pool			1:10	<1:10						

[a]As described in Redfield *et al.*[13]
[b]Six hundred and forty micrograms per injection on days 0, 7, 30, 60, 90, and 120, where day 0 is the study day indicated in the column.
[c]One hundred and sixty micrograms per injection given at varying intervals (2, 4, or 6 months).
[d]Indicates time (post day 0) to generation of new humoral and cellular immune responses to HIV-1 envelope.
[e]Day that antiretrovial therapy was instituted.
[f]Length of follow-up from post-initial day 0 for patients included in viral load analyses.
[g]Trial patients not analyzed in this study: 5, deceased; 22, left the trial; 11 and 12, poor compliance.

primer. The entire reverse transcriptase reaction (100,000 cell equivalents) was used in the 100-μl PCR reaction employing 28 cycles with an annealing temperature of 55°C. As a control for RNA specificity, reactions were also carried out in the absence of reverse transcriptase.

For the quantitative determination of copy number, DNA fragments of HIV-1 target sequences were generated by PCR from the plasmid pBENN 7 and ligated into pGEM-3Z (Promega) or pBluescript II KS (Stratagene, La Jolla, CA) plasmids.[8] For PCR, plasmid DNA was diluted in the presence of carrier tRNA (4 μg/ml) and used directly in the PCR reaction. Amplification was performed exactly as for experimental samples. Products of PCR reactions were analyzed on 1.5% agarose gels, pressure blotted to nylon filters (Stratagene), and probed with ^{32}P end-labeled oligonucleotides specific for sequences internal to the amplified product. Image analysis was carried out using storage phosphor technology (Molecular Dynamics, Sunnyvale, CA). Cell numbers were normalized by comparison with the results of the amplification of β-globin DNA carried out using 7000 cell equivalents with an annealing temperature of 50°C and 22 cycles. All statistical analyses were performed post hoc to the design of experiments and the gathering of PCR data. The Wilcoxon signed rank test was employed for nonparametric statistical comparisons as appropriate. Calculation of the Spearman rank correlation coefficient (rho) was employed in the analysis of the correlation matrix for variables including genomic RNA and *gag* DNA baseline, end-point, and delta values.

Results of PCR analysis reveal several features of the viral load in the cohort of 26 early-stage HIV-infected patients in the phase I trial that are apparent from the descriptive statistics summarized in Table 2. First, there is a three-log range in the level of genomic RNA and a two- to three-log range in *gag* DNA among the patients throughout the study period. This broad range of viral load is characteristic of early-stage HIV-infected persons and

TABLE 2. STATISTICAL ANALYSES OF VIRAL LOAD IN 26 PATIENTS IN GP160 PHASE I TRIAL

Descriptive Facts

Parameter	*Baseline*	*End point*
Geometric mean genomic RNA[a]	75	85
Arithmetic mean genomic RNA	240	247
Range	2–1650	5–1822
Geometric mean *gag* DNA[a]	97	59
Arithmetic mean *gag* DNA	240	203
Range	1–840	1–1175

Statistical Analysis

The results of the Wilcoxon signed rank test show that there is no significant difference between the end point and the baseline as measured for all 26 patients, with $p = 0.56$ for DNA and $p = 0.21$ for RNA

Power: *N* at each time point varies from 1 to 3
Baseline: From 30 days prior to day of vaccination
End point: Last analysis point and up to 60 days previous
Mean follow-up: 702 days; range, 492–812 days

[a]Values are copy number of nucleic acid per 100,000 PBMC

TABLE 3. PROFILE OF CD4+ CELL VALUES IN VIRAL BURDEN STUDY POPULATION

	Absolute CD4+ Cell count			*Percent CD4+ cells*		
Patient	*Baseline*[a]	*End point*[b]	*Delta*	*Baseline*[a]	*End point*[b]	*Delta*
A	601	717	116	27.0	29.7	2.7
B	684	403	−281	32.3	29.0	−5.3
C	422	359	−63	44.0	35.7	−8.3
D	426	355	−71	35.7	28.0	−7.7
E	825	595	−230	30.2	25.7	−4.5
F	420	380	−40	23.3	22.7	−0.6
G	785	626	−159	41.4	41.0	−0.4
H	656	906	251	35.3	37.7	2.3
I	958	718	−240	24.3	20.3	−4.0
J	479	450	−29	24.6	22.7	−1.9
K	758	616	−142	33.4	30.0	−3.4
L	537	365	−171	27.5	23.3	−4.2
M	1433	1239	−194	47.0	45.0	−2.0
N	562	320	−242	23.6	17.7	−5.9
O	762	813	51	43.6	45.7	2.1
P	564	412	−151	28.8	24.7	−4.1
Q	543	505	−38	28.7	26.4	−2.3
R	627	399	−228	30.3	21.0	−9.3
S	1639	1274	−365	55.0	58.0	3.0
T	530	445	−84	30.3	27.7	−2.6
U	438	383	−55	19.8	20.4	0.6
V	752	800	48	44.4	48.3	3.9
W	388	406	18	21.3	20.7	−0.6
X	499	409	−90	29.2	29.0	−0.2
Y	681	567	−114	37.0	34.0	−3.0
Z	407	392	−15	24.8	23.0	−1.8

[a]Baseline includes values at viral burden points up to 70 days prior to immunization.
[b]End point includes values at the final viral burden determination time point, as listed in Table 1, and up to 60 days previous.

TABLE 4. COPY NUMBERS OF NUCLEIC ACID PER 100,000 PERIPHERAL BLOOD MONONUCLEAR CELLS IN PATIENTS IN PHASE I TRIAL[a]

A. Delta Values

	Baseline[b] to day 365[c]		*Day 365[c] to end point[d]*		*Baseline[b] to end point[d]*	
Patient	*DNA*	*RNA*	*DNA*	*RNA*	*DNA*	*RNA*
A	−69	−60	−25	−1510	−94	−1570
B	−264	−109	−556	−242	−820	−351
C	ND[e]	ND	ND	ND	121	247
D	−3	22	5	102	2	124
E	132	39	−192	6	−60	45
F	−25	−1288	−10	−250	−35	−1538
G	612	15	−122	43	490	58
H	46	18	−63	−11	−17	7
I	−12	13	−40	−25	−52	−12
J	−1	11	−8	44	−9	55
K	−56	3	−1	9	57	12
L	−484	36	89	−75	−395	−39
M	−19	8	0	−13	−19	−5
N	137	244	156	10	293	254
O	1	96	−1	−36	0	60
P	133	48	85	1893	218	1941
Q	ND	ND	ND	ND	−368	17
R	−151	247	55	−342	−96	−95
S	3	−16	18	1138	21	1122
T	ND	ND	ND	ND	10	12
U	472	−105	−49	−88	423	−193
V	−22	4	46	19	25	23
W	−16	34	0	−77	−16	−43
X	−264	−140	325	342	61	202
Y	ND	ND	ND	ND	−228	124
Z	1	38	0	175	1	213

B. Actual Values

	At Baseline[b]		*At day 365[c]*		*At end point[d]*	
Patient	*DNA*	*RNA*	*DNA*	*RNA*	*DNA*	*RNA*
A	110	1650	41	1590	16	80
B	840	390	576	281	20	39
C	51	152	ND	ND	172	399
D	20	228	17	250	22	352
E	106	352	238	391	46	397
F	65	1608	40	320	30	70
G	388	185	1000	200	878	243
H	30	11	76	29	13	18
I	65	29	53	42	13	17
J	215	29	214	40	206	84
K	71	2	15	5	14	14
L	634	44	150	80	239	5
M	35	15	16	23	16	10
N	37	131	174	375	330	385
O	1	5	2	101	1	65
P	507	304	640	352	725	2245
Q	464	28	ND	ND	96	45
R	443	135	292	382	347	40
S	10	57	13	41	31	1179
T	580	10	ND	ND	590	22
U	732	280	1204	175	1155	87
V	98	8	77	12	123	31
W	17	83	1	117	1	40
X	344	248	80	108	405	450
Y	368	176	ND	ND	140	300
Z	8	63	9	101	9	276

[a]Values are the average (*N* varies from 1 to 3) number of nucleic acids per 100,000 PBMCs.
[b]Baseline is from 30 days prior to first rgp 160 vaccination.
[c]Day 365 is 365 ± 60 days from day 0.
[d]End point is last day of analysis and up to 60 days previous.
[e]ND, Not determined.

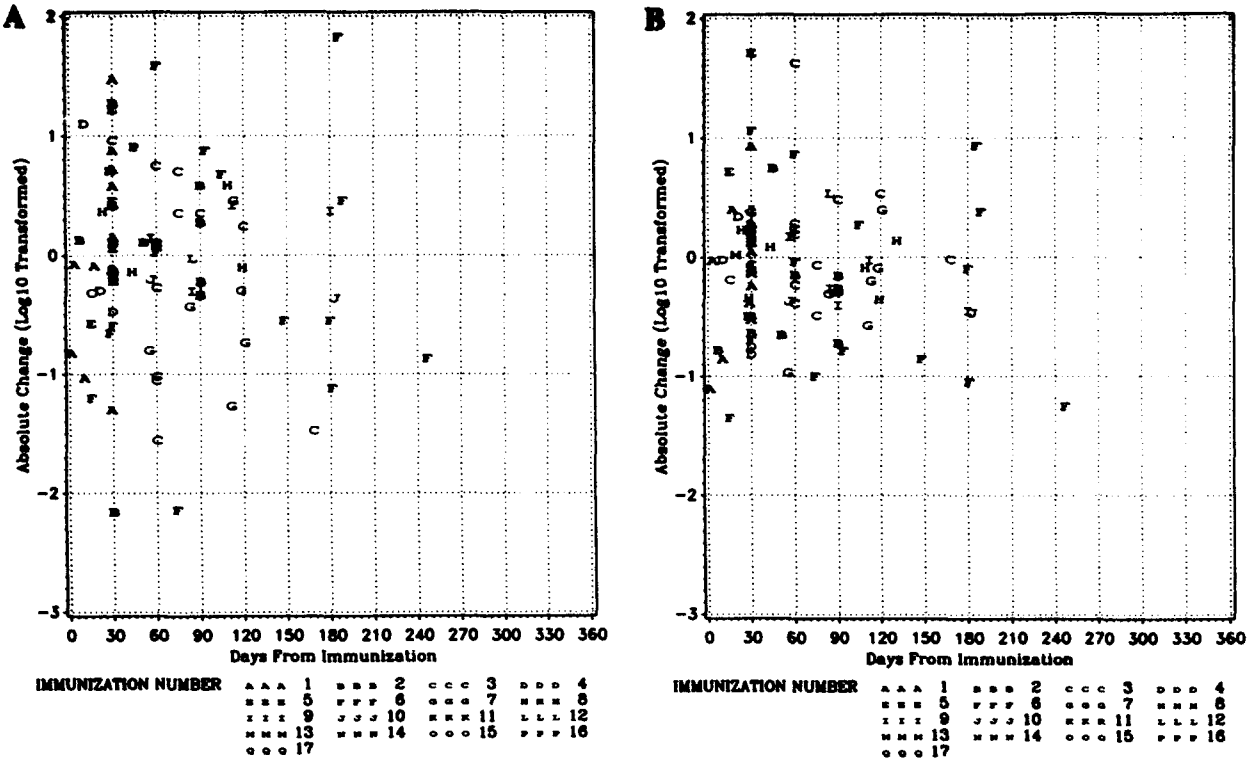

FIG. 1. Distribution of viral burden parameters with immunization points. Time points on the abscissa indicate days since immunization from a reference point of day 0 prior to any immunization. Values on the ordinate are $\log_{10}$-transformed data derived from the absolute change in viral load (expressed as copy number per 100,000 PBMCs) from each immunization for each of the 26 patients. The alphabetic key below the graph indicates the vaccination number. Although the distribution of points appears to be random, unknown cumulative and/or lag effects, as well as placement and the frequency of PCR data points, place qualifications on the data, as discussed in text. (A) Fluctuations in genomic RNA with immunizations; (B) Fluctuations in *gag* DNA with immunizations.

is consistent with our observations made in a cross-sectional study of HIV-infected persons employing these same methodologies.[8] Second, there is no statistically significant increase or decrease in the levels of genomic RNA or *gag* DNA over the study period ($p = 0.21$ for RNA and $p = 0.56$ for DNA).

Table 3 summarizes the characteristics of the CD4+ cell levels in the study population, which are presented at baseline including values up to 70 days prior to immunization, and at an end point defined by the last date of follow-up by PCR analysis as shown in Table 1 and including values up to 60 days previous to that point. These values are presented to provide a context for the viral load data in terms of a more traditional surrogate marker and are not intended to reflect dynamic changes in the CD4+ status in these patients as the result of immunization with the gp160 subunit vaccine.

Delta values for viral load in the study population are presented in Table 4 and include the copy number per 100,000 PBMCs of genomic RNA and *gag* DNA at time points prior to immunization (baseline), at 365 days (±60 days) beyond the first immunization, and at the last point on which viral load analysis was done (range, 570–810 days). When examined in this manner, additional features of the viral load in the population were observed. Patients exhibiting an increase or decrease in load from prebleed to day 365 may increase or decrease from the 365-day point to the 730-day point. Some patients do exhibit a decline over the entire period (A, B, and F) and others an increase over the study period (C, N, P, T, and Z). However, the majority of patients exhibit periods of increase and decrease with no established trend (D,E,G,H,I,J,K,L,M,O,Q,R,S,U,V,W, and Y).

Correlation analysis of trends in viral load parameters, although hampered by the small size of the population and the lack of an appropriate control group, indicate a qualitative relationship between levels of *gag* DNA at baseline and end point with a Spearman rank correlation coefficient of rho = 0.693. Similar analysis indicates a Spearman rank correlation coefficient of rho = 0.542 for values of genomic RNA at baseline and end point. These rho values are indicative of a modest positive correlation given that a rho value of 1.0 defines an absolute correlation. The baseline for these calculations includes values for viral load for up to 70 days previous to immunization and the end point is defined by the last day of follow-up for viral load analysis and up to 60 days previous. The power of viral load to reflect clinical status in HIV disease awaits characterization of trends in a natural history setting as well as demonstration of trends in double-blind placebo-controlled intervention studies.

Qualitative analysis, demonstrating the lack of relationship between immunization and the changes in viral load, is demonstrated in Fig. 1, a scattergram of the fluctuations in *gag* DNA or genomic RNA in relationship to immunization point. Several qualifications must be placed on the interpretation of this data

set. The temporal placement, frequency, and power of the DNA and RNA PCR samples were not amenable to statistical analyses of the relationship between immunization and viral load and only qualitative statements can be made concerning such observations. Furthermore, because parameters of lag and/or cumulative effects of the vaccine are not known in regard to virus load, any statements as to the relationship between immunization and fluctuations in viral load must be considered with these caveats in mind.

As these studies indicate, the validation of the polymerase chain reaction to assess viral load in clinical samples awaits application of such analyses to ongoing double-blind placebo-controlled studies. In this setting more meaningful conclusions may be drawn as to the potential of this technology to define viral burden as an acceptable surrogate marker in HIV clinical disease progression as well as to provide insight into the relationships between viral load and the immune response in interventional studies. Any conclusions as to the long-term safety and/or efficacy of the gp160 vaccine therapy regimen must await the results of such placebo-controlled trials.

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